Chapter 9

Cholesterol and Amyloid β Fibrillogenesis

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Abstract: Evidence is accumulating to suggest that cholesterol is a potent risk factor for the development of Alzheimer's disease. An increase in cholesterol level in neuronal membranes may facilitate the generation and aggregation of the amyloid β-protein (Aβ). Our results and those of other groups suggest that cholesterol has both direct and indirect effects of acceleration of Aβ fibrillogenesis. A novel concept of cholesterol neurobiology is necessary to elucidate the mechanism underlying cholesterol-dependent Aβ pathology.

Key words: Amyloid β-protein, fibrillogenesis, cholesterol, GM1 ganglioside, lipid raft, senile plaque, aging, apolipoprotein

1. INTRODUCTION

Amyloid β-protein (Aβ) deposition in the brain is one of the fundamental processes in the development of Alzheimer's disease (AD); however, the molecular mechanism underlying the conversion of Aβ from its nontoxic soluble form to its toxic aggregated form remains to be determined. Aβ, a naturally occurring proteolytic (β-secretase) derivative of the amyloid precursor protein (APP), is believed to self-aggregate. Indeed, Aβ forms amyloid fibrils following its incubation *in vitro* at concentrations higher than those in physiological biological fluids. However, Aβ aggregation *in vitro* can be significantly suppressed by subjecting an Aβ solution to "de-seeding". In the case of familial AD, the expression of genes responsible for AD is likely to enhance Aβ deposition through accelerated Aβ generation.

However, no evidence has ever been reported to indicate that Aβ generation is perturbed in sporadic AD, a major form of the disease. Thus, it is reasonable to assume that Aβ deposition in the brain is induced by not only an increase in Aβ concentration in cells and/or extracellular space but also as yet unknown environmental factors. Recently, evidence is accumulating to suggest that cholesterol is a potential risk factor for AD development (Simons *et al.*, 2001; Puglielli *et al.*, 2003). In regard to the possible role(s) of cholesterol in the acceleration of Aβ deposition *in vivo*, several investigators have reported that processes in Aβ generation, including APP cleavage by β- and γ-secretases, can be facilitated by an increase in cellular cholesterol concentration (Wahrle *et al.*, 2002; Fassbender *et al.*, 2001; Frears *et al.*,1999; Simons *et al.*, 1998). Alternatively, we and other groups have suggested that Aβ fibrillogenesis is also modulated through direct and indirect interactions with cellular lipids including cholesterol (McLaurin *et al.*, 2002; Yamazaki *et al.*, 2001; Yip *et al.*, 2001; Kakio *et al.*, 2001; Mizuno *et al.*, 1999; Mizuno *et al.*, 1998).

2. CHOLESTEROL AS A RISK FACTOR FOR AD DEVELOPMENT

Previous studies revealed that a number of genetic risk factors are involved in AD development. Notably, among them, there are many genes that encode proteins directly involved in the regulation of lipid metabolism. Apolipoprotein E (ApoE) is a major lipoprotein in the central nervous system. Moreover, it is generally accepted that the genetic polymorphism of the *ApoE* gene is closely related to the prevalence of AD (Corder *et al.*, 1993; Poirier *et al.*, 1993; Saunders *et al.*, 1993; Strittmatter *et al.*, 1993). Much effort has been exerted to elucidate the pathogenic effect of one of ApoE isoforms, ApoE4, the gene product of ApoE allele ε4, the presence of which is a strong risk factor for AD. However, it still remains to be determined how ApoE4 accelerates the progression of AD at molecular and cellular levels. We have been attempting to clarify this question from a viewpoint of the physiological function of ApoE, that is, regulation of cholesterol metabolism in the central nervous system (Michikawa and Yanagisawa, 1998). We have reported that ApoE regulates the turnover of cholesterol and other lipids through the modulation of influx and efflux of

these lipids in an isoform-dependent manner (Gong *et al.*, 2002; Michikawa *et al.*, 2000). Recently, we have also found that ApoE modulates the cholesterol distribution in neuronal membranes as discussed below (Hayashi *et al.*, 2002).

Since the relationship between the presence of ApoE allele ε4 and the prevalence of AD has been confirmed in various ethnic groups, a number of investigators have paid attention to the question as to whether cholesterol metabolism is perturbed in the central nervous system of individuals with AD. Jarvik and colleagues reported that the role of ApoE allele ε4 in the acceleration of AD development is dependent on serum total cholesterol (TC) level as well as age and sex (Jarvik *et al.*, 1995). Additionally, Notkola *et al.* (1998) performed a longitudinal study and concluded that an increase in serum TC level in midlife can be a risk factor for the development of late-life AD. This possibility was supported by the results of Kivipelto *et al.* (2001) who also confirmed that there was a relationship between the level of midlife hypercholesterolemia and the prevalence of late-life mild cognitive impairment (MCI), a putative preclinical stage of AD. Recently, two groups have independently performed retrospective epidemiological studies and reported that statin, an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase, a key enzyme in cholesterol synthesis, has a beneficial inhibitory effect on suppression of AD development (Jick *et al.*, 2000; Wolozin *et al.*, 2000), *see also* Chapter 19.

Previous studies using animal models of AD also suggested that cholesterol can be a risk factor for AD development. Sparks *et al.* (1994) reported that a high-cholesterol diet potentially induces an increase in Aβ immunoreactivity in the brain (Sparks *et al.*, 1994). However, it remains to be clarified how the oral intake of cholesterol can alter the metabolism of lipids and proteins in the central nervous system. Although the accelerated Aβ deposition in the brain induced by high-cholesterol diet requires an explanation, the result of Sparks and collelagues has been supported by recent studies by Refolo *et al.* (2000) who reported that hypercholesterolemia induced by high-cholesterol diet accelerated the Aβ deposition in the brain of APP-transgenic mouse model of AD. They also reported that a cholesterol-lowering drug that inhibits 7-dehydrocholesterol-reductase, the enzyme working upstream of cholesterol biosynthesis, can reduce Aβ deposition in the brain of the same transgenic mouse model (Refolo *et al.*, 2001). These lines of evidence suggest that cholesterol can be transported from plasma to the brain and alter Aβ pathology.

3. CHOLESTEROL AND Aβ FIBRILLOGENESIS

3.1 Direct effect

The interaction of Aβ with plasma membranes is an important subject that needs elucidation. Wood and his colleagues previously examined the possibility of the direct binding of Aβ to lipids, including cholesterol, phosphatidylcholine and fatty acids (Avdulov *et al.*, 1997). They found that Aβ binding to lipids is dependent on the aggregation state of Aβ, that is, monomeric Aβ does not bind to lipids and only aggregated Aβ binds to lipids in a lipid class-dependent manner. Alternatively, McLaurin and her colleagues investigated whether Aβ-lipid interaction is modulated by cholesterol level in membranes using various techniques (Yip *et al.*, 2001). They reported that the extent of Aβ binding to the surface of cultured PC 12 cells decreases with increasing cholesterol content in the cells and that cholesterol-depleted cells had a higher extent of Aβ-cell surface binding. Müeller and his colleagues found that cholesterol content in neuronal membranes negatively correlates with the membrane perturbing effect of Aβ (Kirsch *et al.*, 2002). Taken all together, it is highly likely that Aβ interaction with neuronal membrane is significantly modulated by cholesterol content in such membranes. However, in regard to the potency of cholesterol to accelerate or inhibit Aβ fibrillogenesis, previous studies have caused controversy. Harris investigated how Aβ fibrillogensis *in vitro* is modulated in the presence of cholesterol by transmission electron microscopy using aqueous suspensions of microcrystalline cholesterol and other lipids (Harris, 2002). He found that the marked acceleration of Aβ aggregation is induced in the presence of cholesterol and cholesterol-containing liposomes. Furthermore, Harris suggested that the potentiation of Aβ polymerization is likely to be induced by the hydrophobic interaction between the amino acid side chains of Aβ and the tetracyclic sterol nucleus. In contrast, McLaurin and her colleagues suggested on the basis of the result obtained from *in situ* atomic force microscopy and *ex situ* electron microscopy that bilayer cholesterol content in total brain lipid extracts inversely correlates with the extent of Aβ insertion into the membranes and subsequent Aβ fibrillogenesis on the membrane surface (Yip *et al.*, 2001). Sui and his colleagues focused on Aβ insertion into the membranes and they suggested that the extent of Aβ insertion into lipid bilayers rather increases as cholesterol content increases and such insertion subsequently inhibits Aβ fibrillogenesis on the membrane surface (Ji *et al.*, 2002). Thus, although much effort has been exerted in elucidating the direct effect of cholesterol content on Aβ fibrillogenesis, the results reported to date are still controversial. It is very difficult at this point to conclude whether an increase

in the cholesterol content of neuronal membranes directly accelerates or inhibits Aβ fibrillogenesis.

3.2 Indirect effect

3.2.1 Identification of GM1 ganglioside-bound Aβ

To elucidate the molecular mechanism underlying the initiation of Aβ aggregation in the brain, we previously attempted to identify an Aβ species that initially accumulates in the brains of AD patients (Yanagisawa *et al.*, 1995). We performed sucrose density gradient fractionation of cerebral cortices. We prepared a fraction with the density of the plasma membrane (membrane fraction), which was not likely to contain aggregated Aβ, as well as a fraction with the protein density (amyloid fraction), which was enriched with amyloid cores of senile plaques. We performed Western blot analyses of such fractions using various anti-Aβ monoclonal antibodies, including antibodies specific to the carboxyl- and amino-terminals, and midportion of Aβ. On the blot of the fractions prepared from AD and Down's syndrome (DS) patients in the advanced stage of AD, strong immunoreactivities of those antibodies were observed in the amyloid fraction with a molecular weight of 4 KD. No Aβ immunoreactivity was detected in any fractions prepared from control non-demented individuals, whose cerebral cortical sections did not show Aβ deposition. In contrast, a unique Aβ immunoreactivity was observed in the membrane fractions prepared from the brains which exhibited early pathological changes of AD, including diffuse amyloid plaques. Briefly, Aβ was only detected by antibodies specific to the carboxyl- and amino-terminals of Aβ but not by antibodies specific to the midportion of Aβ. Aβ also showed smearing on the blot with retarded mobility on the gel compared with synthetic Aβ. To determine that the generation of this unique Aβ species is associated with the early pathological stage of AD, we examined a number of brains, including those of AD and DS patients, and nondemented individuals. We performed quantitative densitoscanning of the Western blots and neuropathological analyses of cerebral cortices. The Aβ immunoreactivity ratio of the amyloid fraction to the membrane fraction was determined for each case. The level of diffuse plaque formation was used as a marker of the early stage of AD pathology whereas the level of neurofibrillary tangle formation as that of the advanced stage of AD pathology. From this assessment, the Aβ imunoreactivity ratio of the amyloid fraction to the membrane fraction is positively and negatively correlates with the level of neurofibrillary tangle formation and the level of diffuse plaque formation, respectively. These results suggest that membrane-bound Aβ is preferably generated in the early stage of AD.

Based on the unique molecular features of membrane-bound Aβ, including the absence of the immunoreactivity of anti-Aβ antibodies, which were specific to the midportion of Aβ, smearing and retarded mobility on the gel, we hypothesized that a small molecule, such as a lipid, is bound to Aβ. To examine this possibility, we performed delipidation using various organic solvents, including methanol, ethanol, ether, and acetone. Interestingly, only treatment with methanol markedly altered the features of Aβ, that is, immunoreactivity of the antibody specific to the midportion of Aβ was recovered, and the smearing and retarded mobility of Aβ disappeared after methanol treatment. Since a lipid with a charge is sensitive to methanol, we expected that ganglioside is bound to Aβ in the membrane fraction. To confirm this possibility, we incubated the blot of the membrane fraction with cholera toxin, a specific ligand to GM1 ganglioside. Notably, Aβ in the membrane fraction was labeled with cholera toxin. This binding of cholera toxin to Aβ was lost following methanol treatment. Alternatively, to determine the molecular species of Aβ, we performed Western blot analysis using antibodies specific to Aβ40 and Aβ42. Aβ in the membrane fraction was only recognized by the antibody specific to Aβ42. To determine the amino terminus of the Aβ, we performed mass spectrometry following peptidase treatment. Based on the results of these experiments, we concluded that the Aβ species that accumulated in the membrane fraction is Aβ1-42. To further confirm that Aβ1-42 is generated in the membrane fraction of the brain, we attempted to immunoprecipitate Aβ from the membrane fraction prepared from the AD brain. In this experiment, Aβ1-42 in the GM1 ganglioside-bound form was successfully precipitated (Yanagisawa *et al.*, 1998). Interestingly, in the experiment of immunoprecipitation, the antibody specific to the midportion of Aβ failed to precipitate Aβ from the membrane fraction. This result supports our hypothesis that Aβ adopts an altered conformation through binding to GM1 ganglioside. To further confirm the alteration of Aβ conformation through its binding to GM1 ganglioside, we attempted to generate a monoclonal antibody specific to the GM1 ganglioside-bound form of Aβ (GAβ) (Yanagisawa *et al.*, 1997). Because of the limitated amounts of Aβ obtained from cerebral cortices, we performed *in vitro* immunization. The IgM monoclonal antibody (4396) was obtained by this method. MAb 4396 specifically recognized the Aβ species in the membrane fractions prepared from the brains which exhibited early Aβ and the Aβ recovered in the amyloid fraction prepared from AD brains. pathological changes of AD. However, it did not react with synthetic soluble In binding analysis, MAb 4396 recognized Aβ bound to GM1-containing liposomes. These results strongly suggest that GAβ adopts a conformation distinct from those of soluble and aggregated Aβs. Based on its unique molecular characteristics, including its extremely high aggregation potential

and altered immunoreactivity, we concluded that Aβ binds to GM1 ganglioside at the early stage of AD and adopts an altered conformation, and subsequently accelerates the aggregation of soluble Aβ by acting as a seed. Following our initial report, several investigators performed *in vitro* experiments to confirm the potency of GAβ to serve as a seed. Surewicz and his colleagues prepared liposomes with or without GM1 ganglioside (Choo-Smith and Surewicz, 1997; Choo-Smith *et al.*, 1997). They incubated soluble Aβ with liposomes in a buffer with physiological ionic strength to avoid the non-specific binding of Aβ to liposomes, which is likely to occur under a condition of low ionic strength. They reported that soluble Aβ in the presence of GM1 ganglioside aggregates to form amyloid fibrils (Choo-Smith *et al.*, 1997). These results, taken together with our previous finding, strongly suggest that GAβ accelerates the aggregation of soluble Aβ by acting as a seed.

3.2.2 Cholesterol-dependent generation of GAβ

It remains to be determined how GAβ is generated *in vivo*; however, we found interesting phenomena in our previous studies, that suggested dependence of GAβ generation on cholesterol level (Mizuno *et al.*, 1998; Mizuno *et al.*, 1999). We investigated the molecular mechanism underlying the generation and secretion of Aβ using MDCK cells, which had been extensively studied in terms of cell polarity. We analyzed the Aβ species secreted into the apical and basolateral compartments from MDCK cells. Interestingly, in that experiment, we detected an Aβ species, only in the apical compartment, with unique molecular characteristics shared with GAβ, such as its appearance as a smear on a blot and its altered immunoreactivity. In regard to the mechanism underlying the generation of this unique Aβ species that was secreted into the apical compartment, apical Aβ we hypothesized that cholesterol and/or glycosphingolipids play an important role since these lipids are abundant in apical surface membranes as well as in apically transported vesicles (Simons and Ikonen, 1997). To examine this possibility, we treated cultured MDCK cells with compactin, an inhibitor of cholesterol synthesis. The cholesterol level in the cells decreased to 40% of the control level following treatment with compactin at concentrations ranging from 500 to 1000 nM. The treatment of the cells with compactin at concentrations higher than 5000 nM caused marked cell death, whereas cell death caused by treatment with compactin in the concentrations ranging from 500 to 1000 nM was negligible. Notably, the compactin treatment of the cells significantly decreased the extent of apical Aβ generation. This result suggests that apical Aβ generation is dependent on cholesterol level in membranes. The apical Aβ observed as a smear on immunoblots disappeared

dramatically following treatment of cells with compactin. Since Aβ smearing suggested the potency of apical Aβ to act as a seed for the fibrillogenesis of soluble Aβ, we performed a thioflavin T (ThT) assay of the incubation mixture containing synthetic soluble Aβ and apical Aβ immunoprecipitated from the conditioned media of the apical compartment of the cell cultures. The fluorescent intensity of ThT increased without a lag phase and reached equilibrium hyperbolically in the presence of apical Aβ. In contrast, the ThT fluorescence intensity of the solution of synthetic Aβ in the presence of the Aβ immunoprecipitated from the conditioned media of the basolateral compartment did not increase. To further confirm the acceleration of the fibrillogenesis of soluble Aβ by apical Aβ, we performed electron microscopy analysis. Typical amyloid fibrils were observed in the incubation mixture containing synthetic and apical Aβs. Notably, apical Aβ lost its potency to act as a seed following the treatment of the cells with compactin. These results suggest that Aβ with a seeding activity is endogenously generated in a cholesterol-dependent manner by the cells.

Based on the finding that a seed Aβ is endogenously generated in a cholesterol-dependent manner, we then extended our study to investigate whether GAβ generation is also dependent on cholesterol level (Kakio *et al.*, 2001). In this study, we first prepared liposomes with lipids, *e.g.* cholesterol, phosphatidylcholine, phosphatidylserine, sphingomyelin and GM1 ganglioside of various concentrations. To avoid the nonspecific binding of Aβ to lipids under a low-ionic strength condition, we incubated liposomes with soluble Aβ in an incubation buffer containing 150 mM NaCl. We labeled synthetic Aβ with 7-diethylamonocoumarin-3-carbonyl group (DAC) at its amino terminus. The binding of DAC-labeled Aβ to liposomes was estimated by determining the fluorescence intensity from DAC. With this system, we found that soluble Aβ specifically binds to liposomes containing GM1 ganglioside. Other groups previously reported that soluble Aβ binds to phospholipid vesicles that lack ganglioside; however, they used an incubation buffer with a low ionic strength. Thus, as Surewicz and his colleagues clearly pointed out, it is highly likely that Aβ binding to ganglioside-free liposomes at a low ionic strength is due to nonspecific, purely electrostatic interaction between Aβ and lipids. We then examined how the lipid composition of liposomes modulates Aβ binding to GM1 ganglioside-containing liposomes. We found that Aβ binding is dependent not only on the concentration of GM1 ganglioside but also on cholesterol concentration in a given liposome. Interestingly, the effect of cholesterol concentration in the liposomes on the acceleration of Aβ binding was more marked in the case of a GM1 ganglioside-poor system (20% molar ratio) compared with that in a GM1 ganglioside-rich system (40% molar ratio). These suggest the following: first, GAβ generation *in vivo* likely occurs in

GM1 ganglioside- and cholesterol-rich environments; second, cholesterol plays a critical role in the regulation of Aβ binding to GM1 ganglioside *in vivo* since the concentration of GM1 ganglioside in the brain cannot be as high as 40 mol% in neuronal membranes. To elucidate the molecular mechanism underlying the cholesterol-dependent acceleration of Aβ binding to GM1 ganglioside, we labeled GM1 ganglioside with BODIPY, a well-known fluorophore, to form an excimer that emits redshifted fluorescence compared with monomers through collision of two dye molecules. The fluorescence emission spectra of BODIPY-GM1 ganglioside were recorded using various liposomes. We found that excimer fluorescence increases in a cholesterol-dependent manner in liposomes with a constant concentration of GM1 ganglioside. These results suggest that GM1 ganglioside forms a cluster in a cholesterol-rich environment (Figure 1).

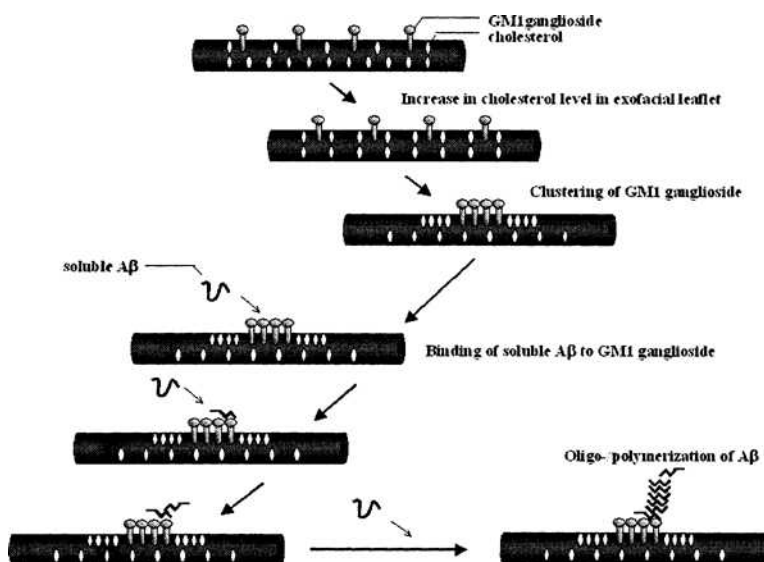

Figure 1. Hypothetical model of cholesterol-dependent oligo-/polymerization of Aβ through GAβ formation on GM1-ganglioside cluster in cholesterol-rich environment.

Recently, evidence has been growing to suggest that APP processing occurs in cholesterol- and GM1 ganglioside-rich microdomains, lipid rafts, in the cells (Ehehalt *et al.*, 2003). It has been suggested that β-cleavage, which is an initial step in Aβ generation from APP, is closely associated with lipid rafts (Fassbender *et al.*, 2001; Frears *et al.*, 1999). Moreover, γ-

cleavage, which is the next step to β-cleavage, has also been recently suggested to be cholesterol-dependent (Wahrle *et al.*, 2002). Taken together with results of our previous studies, it is intriguing to speculate that Aβ generated in lipid rafts or raft-like microdomains start to aggregate following binding to GM1 ganglioside induced by as yet unknown alteration of the composition of lipids, including cholesterol and GM1 ganglioside, of the lipid rafts. This possibility is supported by the following studies: Firstly, Sawamura *et al.* (2000) previously reported that insoluble Aβ accumulates in the fraction of lipid rafts in the brains of the transgenic mouse model of AD. Secondly, Molamder-Melin and colleagues have recently analyzed lipids in the lipid rafts obtained from human brains and suggested that lipid composition is significantly altered in the lipid rafts obtained from AD brains (Molander-Melin *et al.*, 2003). Several investigators previously observed intracellular Aβ deposition prior to the extracellular formation of senile plaques (Gyure *et al.*, 2001; Gouras *et al.*, 2000). With this information, it is interesting to examine whether intracellularly accumulated Aβ is colocalized with marker molecules of lipid rafts, including flottilin and/or GM1 ganglioside. Alternatively, Aβ aggregation induced by GAβ may occur after the shedding of GAβ into extracellular space. In this regard, it is interesting to note that cerebral amyloid can be seeded *in vivo*. Kane et al. previously reported that the inoculation of a dilute AD brain extract induces Aβ deposition in the brains of APP-transgenic mice (Kane *et al.*, 2000).

Matsuzaki and his colleagues have recently investigated how the interaction of Aβ with ganglioside is modulated by ganglioside species (Kakio *et al.*, 2002). They prepared liposomes with a lipid composition similar to that of lipid rafts, containing cholesterol, sphingomyelin and ganglioside. They used major gangliosides in the central nervous system, including GM1, GD1a, GD1b and GT1b. Interestingly, the binding of Aβ to ganglioside was in the order of GM1<GD1b<GD1a=GT1b, suggesting that the number of sialic acid residues plays a critical role in inducing Aβ binding. However, GM1 exhibited the strongest potency to induce the generation of a seed for Aβ fibrillogenesis among the gangliosides examined. GM1 ganglioside is physiologically expressed on the surface of neurons, particularly on synaptic plasma membranes. It is likely that GM1 ganglioside plays a critical role in the modulation of neuronal function and viability. Thus, one could assume that Aβ binding to GM1 ganglioside exerts adverse effects on neurons. McLaurin and Chakrabartty previously reported that Aβ interaction with lipids, including ganglioside, induces membrane disruption (McLaurin and Chakrabartty, 1996). Furthermore, Matsuzaki and Horikiri investigated how Aβ binding to ganglioside affects the structure of the membranes and found that Aβ interaction with ganglioside-containing

such membranes induces the dehydration of lipid interfacial groups and the perturbation of the acyl chain orientation, suggesting that Aβ imposes a negative curvature strain on ganglioside-containing lipid bilayers (Matsuzaki and Horikiri, 1999). Taken together, all these results suggest that, aside from accelerating Aβ fibrillogenesis, Aβ binding to GM1 ganglioside directly suppresses neuronal viability through membrane damage.

3. CHOLESTEROL AND Aβ DEPOSITION *IN VIVO*

The deposition of Aβ in the brain as senile plaques is a fundamental process in AD. However, the precise mechanism underlying the formation of senile plaques remains to be determined. Probst et al. previously suggested that Aβ initially deposits on neuronal plasma membranes (Probst *et al.*, 1991). Yamaguchi et al. performed careful immunohistochemical analysis to clarify the ultrastructural localization of Aβ in diffuse plaques of brains with hereditary cerebral hemorrhage with amyloidosis and AD (Yamaguchi *et al.*, 2000). From their results, they suggested that Aβ starts to deposit in its membrane-bound form. These results suggest that the initial deposition of Aβ occurs on the neuronal surface although it remains to be determined how Aβ adsorbs onto lipids of neuronal membranes. Taken together with results of our studies, one of the possible scenario is that Aβ binds to GM1 ganglioside on the neuronal surface and accelerates the aggregation of soluble Aβ.

Alternatively, Mori *et al.* (2001) directly examined whether cholesterol is associated with senile plaques. They found the abnormal accumulation of cholesterol in cores of mature plaques but not in diffuse or immature plaques in AD brains. They also examined the transgenic mouse model of AD and obtained an essentially similar result. The results of Mori and colleagues suggest that cholesterol plays a role in formation, progression and/or stabilization of senile plaques.

With regard to the initial site of Aβ deposition in the brain, Ihara and his colleagues performed immunochemical analysis of human prefrontal cortices and the brains of the transgenic mouse model of AD (Oshima *et al.*, 2001). They fractionated the cortices by sucrose density gradient centrifugation and then isolated detergent-insoluble low-density membrane (LDM) fractions. They determined Aβ level in those fractions and found that Aβ associated with LDM fractions reflected the extent of the level of extracellularly deposited Aβ. This result, together with the result of their previous study (Sawamura *et al*, 2000), suggests that Aβ initially deposits in LDM that is likely to be cholesterol- and GM1-ganglioside-rich microdomains.

4. CHOLESTEROL IN BRAIN AND NEURONAL MEMBRANES

Cholesterol is one of major lipids in cellular membranes and is essential for many cellular functions. This is also the case for cholesterol in the brain; however, little is known about cholesterol metabolism in the brain. The brain is highly enriched in cholesterol. It accounts for only 2% of the whole body mass but contains almost one-quarter of the unesterified cholesterol in the whole body (Dietschy and Turley, 2001). Although recent study provided the possibility of cholesterol transport across the blood-brain barrier (Refolo *et al.*, 2000), it has been generally accepted that cholesterol turnover is restricted only inside the brain, at a very low rate. Only 0.02% of the cholesterol pool in the brain undergoes a turnover each day (Dietschy and Turley, 2001). This is less than 1% as fast as the cholesterol turnover in the whole body (Dietschy and Turley, 2001). These lines of evidence imply that function and metabolism of cholesterol in the brain is unique. Again, although the results of recent study suggest that cholesterol input from plasma into the brain is not negligible (Refolo *et al.*, 2000), most cholesterol in the brain is likely to come from *in situ* synthesis. Interestingly, the rate of *in situ* cholesterol synthesis in many organs, including nervous system, depends on age (Cenedella and Shi, 1994; Stahlberg *et al.*, 1991; Goodrum, 1990; Popplewell and Azhar, 1987). In general, the rate of cholesterol synthesis is high in fetuses and newborn animals and decreases with age. Furthermore, Vance *et al.* (1994) previously reported that cholesterol is the only major lipid in the brain that cannot be synthesized at the end of neurites, implying that cholesterol level at the ends of neurites depends on two inputs, including axonal flow from the soma and uptake from the extracellular space through apolipoprotein receptors. Thus, it is reasonable to assume that the functions of neurons in the elderly depend on exogenous cholesterol level to a higher degree than those in young individuals. Aging is the strongest risk factor for the AD development, thus, it is interesting to speculate the pathological significance of aging for AD from the viewpoint of the aging-associated alteration of the synthesis and transport of cholesterol.

Neurons have a large plasma membrane compared with any other cell types. Moreover, part of the neuronal surface is specialized to integrate neuron-specific functions such as synaptic transmission. Cholesterol accounts for over 40 mol% of the total membrane lipids in synaptic plasma membranes (Schroeder *et al.*, 1991; Wood *et al.*, 1989). Thus, it is important to clarify how cholesterol in neuronal membranes, particularly in synaptic plasma membranes, is regulated. Wood and his colleagues and other groups have exerted much effort to clarify this issue and suggested the following (Wood *et al.*, 1999): First, cholesterol is not evenly distributed throughout

neuronal membranes but is located in different pools, including cholesterol lateral domains and transbilayer cholesterol domains. In regard to cholesterol lateral domains, there seem to be two distinct pools: cholesterol in one pool is not stable but rather easily exchangeable following biochemical treatment, including incubation with small unilamellar vesicles, whereas that in the other pool is non-exchangeable. It was previously reported that the acetylcholine receptor is closely associated with the poorly exchangeable pool (Leibel *et al.*, 1987), thus, it is important in future studies to elucidate how the physiological function and pathological changes of neurons are associated with these two types of cholesterol lateral domain. Alternatively, regarding transbilayer cholesterol domains, previous studies have revealed an alteration in cholesterol distribution in the domain in association with the risk factors for AD development, as discussed below (Hayashi *et al.*, 2002; Igbavboa *et al.*, 1997; Igbavboa *et al.*, 1996).

It has been a controversial issue whether the net cholesterol content is altered in the AD brain. Mason *et al.* (1992) previously performed X-ray diffraction analysis of lipid membranes extracted from the cortical gray matter of AD brains. They found that the unesterified cholesterol:phospholipid molecular ratio significantly decreased by 30% in the AD brain compared to that in age-matched controls. Soderberg *et al.* (1992) performed a similar analysis using different brain regions from AD patients. In their experiments, the cortical area affected with AD showed an elevated phosphatidylinositol content; however, cholesterol level was highly variable but mostly unchanged. It seems likely that this controversy stems from the difference in techniques employed to determine lipid level. Another important point noted when we evaluated the data obtained from postmortem tissue is that autopsy samples may show modified the levels of lipids or proteins, which are different from those observed during the disease process.

Pathological changes, including the formation of senile plaques, can be induced in the brains of elderly patients with Down's syndrome (DS). One possible explanation for the AD development in the DS brain is the high expression level of the APP gene by trisomy of chromosome 21. However, previous studies also provided evidence suggesting that lipid metabolism is altered in DS (Scott *et al.*, 1994; Naeim and Walford, 1980). Naeim and Walford reported that a membrane prepared from the peripheral mononuclear cells of DS patients showed an increased rigidity, suggesting the increased cholesterol level in the membrane (Naeim and Walford, 1980). Diomede et al. obtained additional results that supported the conclusion of the earlier study showing the increase in serum cholesterol level in DS. In order to clarify the mechanism underlying the altered lipid metabolism in DS, Diomede and co-workers analyzed liver cholesterol levels and the activities of the sterol regulatory element binding proteins, SREBP-1 and

SREBP-2, which are involved in the regulation of cholesterol synthesis (Diomede *et al.*, 1999). They found that trisomy 21 fetuses show an altered pattern of SREBP activation such as the sterol-independent maturation of SREBP-1. This result suggests that alteration in the regulation of cholesterol synthesis is induced by trisomy 21, resulting in high circulating and tissue cholesterol level.

Recently, novel information on cholesterol metabolism in AD has been obtained by determining 24S-hydroxycholesterol (24S-OH-Chol) level in cerebrospinal fluid. 24S-OH-Chol, an endogenously oxidized metabolite of cholesterol, is almost exclusively produced in the brain (Bjorkhem *et al.*, 1998), that is, the 24S-OH-Chol concentration in the brain is 30-1500-fold higher than those in any other organs. An important feature of 24S-OH-Chol is its potency to pass through the blood-brain barrier. It has been previously suggested that a continuous flux of 24S-OH-Chol across the blood-brain barrier is critical for cholesterol homeostasis in the brain. The plasma 24S-OH-Chol level is dependent on age; the level is high before age 20 and then falls after age 20. Based on the characteristics of 24S-OH-Chol, it is likely that the plasma 24S-OH-Chol level can be a peripheral marker of cholesterol metabolism in the brain. Lütjohann *et al.* (1996) performed determination of the plasma 24S-OH-Chol level in AD, non-AD demented and depressive patients and in healthy controls. The plasma 24S-OH-Chol levels in the AD and non-AD demented patients was elevated modestly but significantly compared with depressive patients and healthy controls. There was no significant difference in the 24S-OH-Chol levels between the AD and non-AD demented patients. Importantly, plasma 24S-OH-Chol level negatively correlated with the severity of dementia. This finding was supported by the study of Papassotiropoulos *et al.* (2000). The inheritance of the apoE4 allele is associated with the reduced plasma 24S-OH-Chol level in a manner independent of the severity of dementia in the patients examined. It has also been reported that the 24S-OH-Chol level was elevated in the cerebrospinal fluid in patients with mild cognitive impairment (MCI), an early stage of AD, and AD (Papassotiropoulos *et al.*, 2002; Schonknecht *et al.*, 2002), suggesting that cholesterol turnover is accelerated in the brain of the patients with AD. It remains to be determined how the plasma 24S-OH-Chol level is elevated in association with AD. In this regard, it is interesting to note that polymorphism in the cholesterol 24S-hydroxylase (CYP46) gene, which encodes enzyme involving in conversion of cholesterol to 24S-OH-Chol, is associated with AD (Papassotiropoulos *et al.*, 2003; Kolsch *et al.*, 2002). A genotype that is more prevalent in the AD group is associated with an increased 24S-OH-Chol/cholesterol ratio in CSF. Taken together, it is possible to assume that the accelerated conversion of cholesterol to 24S-OH-

Chol, which may or may not be with polymorphism of the CYP46 gene, predisposes to AD by altering cholesterol turnover in the brain.

Aging is the strongest risk factor for AD development. Thus, it is important to elucidate whether cholesterol distribution in neuronal membranes is altered with aging. As discussed above, there are two transbilayer cholesterol pools, including the cholesterol pool in exofacial and cytofacial lipid leaflets. Importantly, membrane fluidity and cholesterol distribution differ between these leaflets. A cytofacial leaflet contains seven times as much cholesterol as an exofacial leaflet (Igbavboa *et al.*, 1996). In accordance with this asymmetric distribution of cholesterol throughout the lipid bilayer, a cytofacial leaflet is much less fluid compared with an exofacial leaflet. Wood *et al.* (1999) previously carefully determined the age-dependent alteration of membrane fluidity and cholesterol distribution. They examined the synaptic plasma membranes (SPMs) obtained from mice in three different age groups, namely, 3-4, 14-15, and 24-25 months old. In that experiment, the exofacial leaflet of SPM from young mice was significantly more fluid than the cytofacial leaflet. In contrast, the difference in membrane fluidity between the exofacial and cytofacial leaflets was not significant in the SPM prepared from old mice. There was an approximately two-fold increase in the cholesterol level of the exofacial leaflet in the old mice compared with that in the young mice. Since the total amount of cholesterol in SPM did not change, they concluded that the asymmetric distribution of cholesterol throughout the lipid bilayer of SPM changes with age.

At this point, the mechanism underlying the generation of asymmetry in the cholesterol distribution throughout lipid bilayers remains to be determined. Wood and his colleagues suggested that apoE and the LDL receptor are involved in the regulation of the distribution of cholesterol in the SPM lipid bilayer from the results of their studies (Igbavboa *et al.*, 1997). Using apoE- and LDL-receptor-deficient mice, they performed experiments similar to those on aged mice. Interestingly, the cholesterol level in the SPM exofacial leaflets of the apoE- and LDL-receptor-deficient mice showed a twofold increase compared with that in wild-type mice. The possibility of apoE involvement in the regulation of the cholesterol distribution in the SPM lipid bilayer has been further supported by the results of our recent study. We analyzed the transbilayer distribution of cholesterol in the SPM of human apoE3- and apoE4-knock-in mice (Hayashi *et al.*, 2002). In these experiments, we prepared various subcellular fractions, including plasma membrane and endoplasmic fractions, and determined the levels of phospholipid and cholesterol (total and free) in the fractions. There were no significant differences in lipid concentration in the subcellular fraction among the wild-type, apoE3- and apoE4-knock-in mice. The

asymmetric distribution of cholesterol in the SPM of the apoE3-knock-in mice did not differ from that of the wild-type mice; however, it was significantly altered in the apoE4-knock-in mice. The difference observed in the SPM prepared from the apoE4-knock-in mice was essentially the same as those observed in the aged and apoE-knock-out mice; that is, there was an approximately two-fold increase in the cholesterol concentration in the exofacial leaflet of SPM in the apoE4-knock-in mice compared with those in the other two groups of mice. Concomitantly, there was a significant decrease in the level of cytofacial leaflet cholesterol. The total amount of cholesterol in SPM did not significantly differ among the three mouse groups; thus, we concluded that apoE4 expression induces alteration in the cholesterol distribution throughout the lipid bilayer of SPM, thereby resulting in the exofacial leaflet having a higher cholesterol level than the cytofacial leaflet.

Aging and apoE4 expression are the major risk factors for AD development. Thus, it may be important that these two factors exert the same effect on the cholesterol distribution in SPM. It is also interesting to note that the extent of increase in the cholesterol level of the exofacial leaflet induced by these two factors are essentially the same. This result suggests that there are two pools of cholesterol in the lipid bilayer of SPM; cholesterol level in one pool changes in association with biological factors, including aging and apoE expression, whereas that in the other pool is rather stable.

The possible role of cholesterol in the acceleration of Aβ deposition in the brain has also been suggested by recent studies on Nieman-Pick type C disease (NPC). NPC is an autosomal recessive neurovisceral lipid storage disorder (Pentchev *et al.*, 1995). It is characterized by the abnormal accumulation of free cholesterol in lysosomes. The causative genes, including Niemann-Pick C1 (NPC1), have been identified and evidence is growing to indicate that the abnormal trafficking of exogenous cholesterol is closely associated with the progression of the disease. Previous pathological studies on NPC brains revealed the formation of neurofibrillary tangles, composed of hyperphosphorylated tau, which are indistinguishable from those formed in AD brains (Auer *et al.*, 1995; Suzuki *et al.*, 1995). Recently, it has been reported that Aβ deposition is induced in NPC brains. Murayama and his colleagues performed serial immunohistochemical examination of nine NPC brains (Saito *et al.*, 2002). They found Aβ deposition in the form of diffuse plaques in three of the nine brains. Notably, all the three NPC patients positive for Aβ deposition were below age 40 and were homozygous for apoE allele ε4. At age 40, it is is too early to observe Aβ deposition even in individuals homozygous for apoE allele ε4; thus, it is likely that the expression of the mutated *NPC* gene, combined with homozygosity for apoE allele ε4, is responsible for the induction of Aβ deposition in the brain. At

this point, it remains to be elucidated how abnormal trafficking and/or cholesterol accumulation in lysosomes is associated with Aβ deposition in the brain. In this regard, a report by Sugimoto *et al.* (2001) should be to noted. They investigated the intracellular trafficking of lipids, including cholesterol and GM1 ganglioside, in NPC1-deficient cells. In their experiment, they found the accumulation of not only cholesterol but also GM1 ganglioside in NPC1-deficient cells. Although GM1 ganglioside and cholesterol accumulate in different vesicular compartments of NPC1-deficient cells, that is, cholesterol is accumulated in lysosomes whereas GM1 ganglioside in early endosomes, one can assume that the abnormal accumulation of these two lipids is responsible for Aβ deposition *in vivo.* Yamazaki *et al.* (2001) investigated whether Aβ aggregation is induced in NPC-deficient cells. They treated CHO cells with U18666A, which induces the NPC-mimicking accumulation of cholesterol in the cells. They, then, determined intracellular Aβ level and found that Aβ accumulates in its aggregated form in the late endosomes of the cells in association with intracellular cholesterol accumulation. The accumulated Aβ in the cells disappeared following U18666A withdrawal. Importantly, the level of Aβ secreted into the culture medium was not altered with U18666A treatment. These results suggest that intracellular Aβ accumulation is closely associated with the abnormal accumulation of cholesterol in the cells and that this abnormal Aβ accumulation is not due to accelerated Aβ generation. Recent studies suggest that an increase in cholesterol content potentially upregulates Aβ generation. However, evidence obtained from the NPC model cells suggests that an increase in intracellular cholesterol level facilitates Aβ aggregation through an as yet undetermined process, which is not dependent on accelerated Aβ generation induced by cholesterol.

5. CONCLUSIONS AND PERSPECTIVES

In the past few years, considerable attention has been focused on the possible link between cholesterol metabolism and AD development. However, we are still far from understanding the mechanism underlying the cholesterol-dependent acceleration of the pathological processes of AD. In regard to the putative acceleration of the early phase of amyloid cascade by cholesterol, evidence obtained to date suggests that both the generation and aggregation of Aβ is accelerated in a cholesterol-rich environment (Figure 2).

To further elucidate the pathological significance of cholesterol in Aβ metabolism, we need technological progress that will enable us to visualize cholesterol domains and to monitor cholesterol turnover inside neurons.

Cholesterol is not likely to be evenly distributed, but rather, concentrated in particular domains, including lipid rafts. Only recently, a probe for detecting cholesterol-rich microdomains has been developed. This probe will provide new approaches for the understanding of cholesterol homeostasis in neurons.

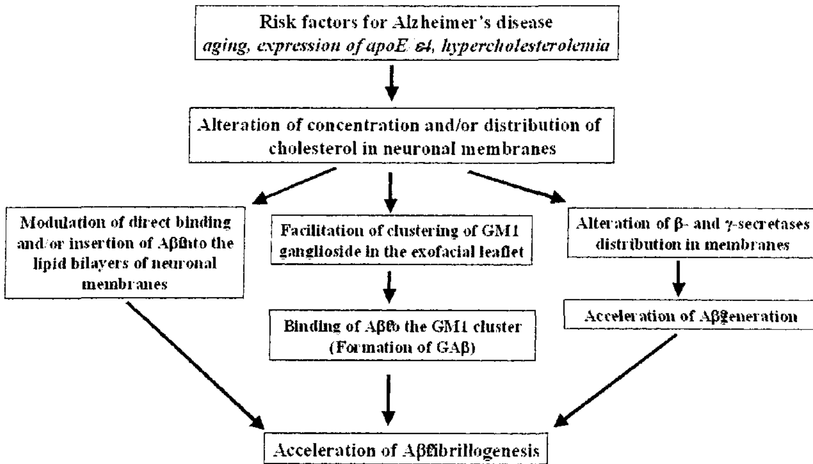

Figure 2. Possible mechanisms underlying cholesterol-dependent facilitation of Aβ fibrillogenesis.

Our studies also show that a decrease in cholesterol content in neurons causes pathological changes, including hyperphosphorylation (*see also* Chapter 20) and apoptosis, that are neuronal pathological features other than Aβ deposition in AD brains. At this point, we do not know whether too much or too little cholesterol is more crucial to AD development. AD is a disease whose pathological processes occur for more than a decade. Thus, it is likely that cholesterol is involved in the pathological process of AD in different manners during the long development of the disease. In conclusion, cholesterol is linked to AD development. A careful functional analysis of cholesterol from both physiological and pathological viewpoints will be helpful in future studies to further our understanding of AD.

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